PB86-212586

Quantitative Characterization of Microbial Biomass and Community Structure in Subsurface Material: A Prokaryotic Consortium Responsive to Organic Contamination

(U.S.) Robert S. Kerr Environmental Research Lab., Ada, OK

19896

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1. REPORT NO. EPA/600/J-86/066	2. PB86-212586
4. TITLE AND SUBTITLE QUANTITATIVE CHARACTERIZATION OF MICROBIAL BIOMASS AND COMMUNITY STRUCTURE IN SUBSURFACE MATERIAL: A PROKARYOTIC CONSORTIUM RESPONSIVE TO ORGANIC CONTAMINATION (Journal Version)	5. REPORT DATE 1986 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) G.A.Smith,J.S.Nickels,B.D.Kerger,J.D.Davis, S.P.Collins--Dpt.of Biological Sci.,Fla. St. Univ.; J.T.Wilson,J.F.McNabb--U.S.EPA,Ada,OK; D.C.White--Fla.State U.	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Biological Science Florida State University Tallahassee, Florida 32306-3043	10. PROGRAM ELEMENT NO. CBPC1A 11. CONTRACT/GRANT NO. CR-809994
12. SPONSORING AGENCY NAME AND ADDRESS Robert S. Kerr Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Ada, OK 74820	13. TYPE OF REPORT AND PERIOD COVERED Journal Art. 1984-85 14. SPONSORING AGENCY CODE EPA/600/15

15. SUPPLEMENTARY NOTES
Project Officer: James F. McNabb
Canadian Journal of Microbiology, Feb.1986, Vol 32, pgs. 104-111

16. ABSTRACT

Application of quantitative methods for microbial biomass, community structure, and nutritional status to the subsurface samples collected with careful attention to contamination reveals a group of microbes. The microbiota are sparse by several measures of biomass compared to surface sediments and soils. The community structure as characterized by the patterns of ester-linked fatty acids from the phospholipids shows an absence of long chain polyenoic fatty acids typical if microeukaryotes and with high proportions of fatty acids typical of bacteria. Subsurface samples contain a higher proportion of glycerol teichoic acids than surface samples. Microbes in uncontaminated subsurface sediments show nutritional stress as evidenced by high levels of poly beta-hydroxybutyrate and extracellular polysaccharides. The ester-linked phospholipid fatty acids show distinctive differences in proportions between surface and subsurface, between subsurface sandy-clay and limestone, and between two sites of subsurface sandy-clay using stepwise discriminant analysis. Contamination increased the microbial biomass, shifted the community to a more gram negative bacterial consortium and induced growth as evidenced by phospholipid fatty acid biosynthesis.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS *(This Report)* unclassified 20. SECURITY CLASS *(This page)* unclassified	21. NO. OF PAGES 9 22. PRICE

EPA Form 2220-1 (Rev. 4-77)

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EPA/600/J-86/066
JOURNAL ARTICLE

Quantitative characterization of microbial biomass and community structure in subsurface material: a prokaryotic consortium responsive to organic contamination

GLEN A. SMITH, JANET S. NICKELS, BRENT D. KERGER, JOHN. D. DAVIS, AND STEPHEN P. COLLINS
Department of Biological Science, Florida State University, Tallahassee, FL, U.S.A. 32306-3043
JOHN T. WILSON AND JAMES F. MCNABB
Robert S. Kerr Environmental Research Laboratory, United States Environmental Protection Agency, Ada, OK, U.S.A. 74820
AND
DAVID C. WHITE[1]
Department of Biological Science, Florida State University, Tallahassee, FL, U.S.A. 32306-3043

Accepted October 22, 1985

SMITH, G. A., J. S. NICKELS, B. D. KERGER, J. D. DAVIS, S. P. COLLINS, J. T. WILSON, J. F. MCNABB, and D. C. WHITE. 1986. Quantitative characterization of microbial biomass and community structure in subsurface material: a prokaryotic consortium responsive to organic contamination. Can. J. Microbiol. **32**: 104–111.

Application of quantitative methods for microbial biomass, community structure, and nutritional status to the subsurface samples collected with careful attention to contamination reveals the presence of a group of microbes. The microbiota is sparse by several measures of biomass compared with that present in surface sediments and soils. The community structure, as characterized by the patterns of ester-linked fatty acids from the phospholipids, shows an absence of long-chain polyenoic fatty acids typical of microeukaryotes and high proportions of fatty acids typical of bacteria. Subsurface samples contain a higher proportion of glycerol teichoic acids than surface samples. Microbes in uncontaminated subsurface sediments show nutritional stress as evidenced by high levels of poly-β-hydroxybutyrate and extracellular polysaccharides. The proportions of ester-linked phospholipid fatty acids show distinctive differences between surface and subsurface, between subsurface sandy clay and limestone, and between two sites of subsurface sandy clay as shown using stepwise discriminant analysis. Contamination increased the microbial biomass, shifted the community to a more gram-negative bacterial consortium, and induced growth as evidenced by phospholipid fatty acid biosynthesis.

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L'application de méthodes quantitatives pour l'étude de la biomasse microbienne, de la structure communautaire et du statut nutritionnel d'échantillons de sous-surface, prélevés avec grand soin face à la contamination, révèle un groupe de microbes. D'après plusieurs méthodes d'analyses, les microbiontes de la biomasse sont rares, par comparaison avec les sédiments et les sols de surface. La structure communautaire, caractérisée par les patterns de liens esters d'acides gras des phospholipides, montre une absence d'acides gras polyénoïques à longues chaînes typiques des microeucaryotes et des proportions élevées d'acides gras typiques des bactéries. Les échantillons de sous-surface contiennent une proportion plus élevée d'acides téichéiques des glycérols que les échantillons de surface. Les microbes de sédiments non contaminés des sous surfaces présentent un stress nutritionnel tel que mis en évidence par les niveaux élevés de poly-β-hydroxybutyrates et de polysaccharides extracellulaires. Les liens esters d'acides gras des phospholipides présentent des différences de proportions notoires entre une surface et une sous-surface, entre sous-surface d'argile sablonneuse et sous-surface calcaire, et entre deux sites de sous-surface d'argile sabonneuse si l'on a recours à une analyse discriminante par étape. Les contaminations font augmenter la biomasse microbienne, favorisent un changement dans la communauté vers un consortium de bactéries davantage Gram négatives et induisent la croissance comme le démontre la biosynthèse d'acides gras phospholipidiques.

[Traduit par le journal]

Introduction

Groundwater is becoming increasingly important as a source of clean, fresh water for industrial and domestic use. At the same time, more of this vital resource is found to be contaminated with potentially toxic wastes. For years the subsurface soil environment that provided the groundwater was assumed to be sterile or to be an unimportant site of microbial activity. Early studies showed a rapid drop-off in the numbers of microbes with depth (Waksman 1916), a nutrient source of organic carbon generally less than 1 mg/L, and a shielding mantle of soil that would protect the groundwater. The pioneering studies of Dunlap et al. (1972) showed anaerobic microbial activity directed against nitriloacetic acid in shallow subsurface aquifers and opened the way to defining the subsurface as a special microbial environment. The research group at the Robert S. Kerr Environmental Research Laboratory at Ada, OK, developed an aseptic sampling technique for unconsolidated subsurface sediments that protected the sediments from contamination with surface microbes (Dunlap et al. 1977). These techniques were improved by the utilization of a sterilized paring device that stripped the outer 1 cm of sediment from the cores to yield essentially uncontaminated subsurface sediments for study (Wilson, McNabb, Balkwill et al. 1983).

Microscopic enumeration and morphological examinations of these sediments showed them to contain a sparse and unusual microbiota (Ghiorse and Balkwill 1983).The importance of the microbial activities of this unusual and sparse microbiota prompted the application of quantitative chemical assays of microbial biomass, community structure, and metabolic activity that are free from the bias of incomplete recovery from sediment granules or the inadequacies of viable culture techniques (White 1983). Biomass can be estimated from cellular components universally distributed in microbes and community structure by detecting "signature" components that are restricted

[1]Author to whom reprint requests should be addressed.

Printed in Canada / Imprimé au Canada

to subsets of the total community. Methods exist or have been developed in this laboratory to detect the following components in subsurface, unconsolidated materials: muramic acid (a unique component of the prokaryotic cell wall) and glucosamine (a component of both prokaryotic and microeukaryotic cell walls) which can be detected after hydrolysis (Findlay et al. 1983); total extractable phospholipid measured as lipid phosphate (White, Davis et al. 1979) or glycerol phosphate (Gehron and White 1983); total ester-linked phospholipid fatty acids (PLFA) (Bobbie and White 1980); neutral lipid glyceride glycerol (Gehron and White 1982); the content of hydroxy fatty acids in the lipopolysaccharide (LPS) – lipid A of gram-negative bacteria (Parker et al. 1982); and the ribitol and glycerol of the teichoic acid polymers in the walls of gram-positive bacteria. Analysis of the ester-linked PLFA by capillary gas–liquid chromatography – electron impact mass spectrometry (cGC–MS) has proven especially useful in defining the microbial community structure. Certain profiles of fatty acids have been shown to be associated with various components of the microbial community (White et al. 1980; Bobbie and White 1980; Bobbie et al. 1981; Parkes and Taylor 1983). High levels of specific ester-linked PLFA appear to be associated with sulfate-reducing bacteria (Edlund et al. 1985; Parkes and Taylor 1983), and plasmalogen phospholipids are associated for the most part with the bacterial anaerobic fermenters (White, Bobbie, King et al. 1979). The methane-forming archaebacteria contain the unique phytanyl glycerol ethers (Martz et al. 1983).

Thus far it has proved impractical to measure microbial activity *in situ* in the subsurface by inoculating labeled precursors and monitoring incorporation or turnover in "signature" components. Consequently measures of nutritional status were developed. Endogenous storage lipids accumulate under conditions where cellular growth is repressed. Hence, sensitive methods for the detection of triglyceride glycerol and wax esters from the microeukaryotes (Gehron and White 1982) and of poly-β-hydroxybutyrate (PHB) from a subset of the prokaryotes were developed (Nickels et al. 1979; Findlay and White 1983). Extracellular polysaccharide glycocalyxes, as measured by their polymer uronic acids (Fazio et al. 1982), accumulate under unfavorable growth conditions (Uhlinger and White 1983). The ratio of the exopolymer or endogenous storage lipids to the phospholipid has proven an especially valuable measure of the nutritional status of the microbiota.

Application of these methods to cores of unconsolidated subsurface material recovered with the aseptic technique has made it possible to demonstrate that a prokaryotic microbiota exists in sandy clay (White et al. 1985). The microbiota is sparse with the equivalent of 10^6–10^7 cells per gram dry weight as compared with 10^9 per gram dry weight in surface soils. The microbiota is essentially prokaryotic with little evidence of microorganisms containing long-chain polyenoic fatty acids ester-linked in the phospholipid fraction. The fatty acid composition of the phospholipids is unusual in comparison with estuarine and abyssal sediments as it is enriched in short-chain branched and normal fatty acids and in monoenoic fatty acids with w 7 unsaturation which are characteristic of the anaerobic desaturase pathway (Bobbie and White 1980). The lipids of the subsurface microbiota appeared to be enriched in the plasmalogen phospholipids typical of anaerobic fermenters (White et al. 1985). The proportions of gram-positive bacteria containing glycerol teichoic fatty acids increase with the depth of uncontaminated sediments (Gehron et al. 1984). There is a high proportion of phosphatidyl glycerol in the phospholipids of the subsurface sediments suggesting an enrichment in prokaryotes (Gehron and White 1983). In uncontaminated aquifer sediments there are indications of great nutritional stress as evidenced by the very high ratios of PHB and glycocalyx per cell. These findings have been confirmed in subsurface sediments recovered aseptically in Florida, Georgia, Texas, Louisiana, and Oklahoma. The same distinctive patterns were detected in aquifer sediments recovered from impermeable clay from a depth of 420 m (White et al. 1983).

In the present study the application of these methods permits the documentation of the differences in the biomass and community structure between the surface and subsurface microbiota in uncontaminated sandy clay soils and subsurface limestones, and further establishes that a subsurface microbiota exists in uncontaminated aquifers. This study further indicates that there are marked shifts that parallel the degree of contamination. In the vadose zone of sediments from Conroe, TX, contaminated with creosote waste, the degree of increase in contamination is reflected in a higher cellular biomass (measured as extractable phosphilipid), a shift from predominantly gram-positive to gram-negative cell types, a distinctive ester-linked PLFA pattern, and possibly an increased rate of PLFA synthesis with a smaller increase in the rate of PHB synthesis from acetate.

Materials and methods

Materials

Baker resi-analyzed glass-distilled solvents and reagents were purchased from Scientific Products, Stone Mountain, GA.

Sampling sites

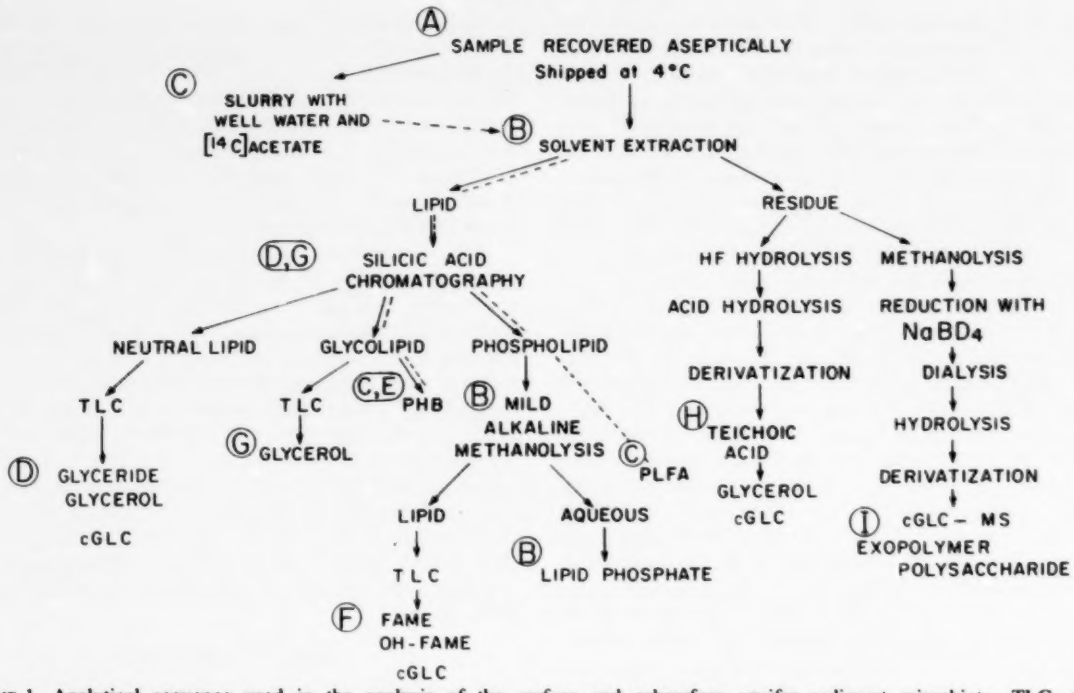
Uncontaminated sandy clay sediments were collected from subsurface sediments to a depth of 10 m from unconsolidated sandy clays, which sometimes contain small gravel deposits, on the flood plain margin of a small river at Lula, OK. The dry surface soil was a rich agricultural soil. Subsurface sandy clays were collected from uncontaminated, contaminated, and a more contaminated site at Conroe, TX, on the Gulf coastal plain from an area covered with a poor and highly weathered soil which overlays alternating beds of sands and clays of meander belts. A filled lagoon was the source of creosote wastes. The wastes penetrated the meander belt and entered deeper transmissive sandy clays. Samples were recovered from both sites from the vadose and saturated zones using the aseptic technique developed at the Robert S. Kerr Environmental Research Laboratory (RSKERL) (Wilson et al. 1983). The sediments were shipped in ice to Florida State University (FSU) and the analysis was begun within 24 h of collection. Samples of buff limestone recovered from Lincolnshire, England, taken at a depth of 37.5 m, were lyophilzed and sent to the FSU laboratory by A. R. Lawrence, Hydrogeology Unit, Institute of Geological Sciences, of the Natural Environmental Research Council, Wallingford, Oxfordshire.

Extraction of the lipids

The lipids were extracted with the single-phase chloroform–methanol method (White, Davis, et al. 1979). The lipids were filtered through fluted Whatman 2V filtered paper and stored under nitrogen at −2°C.

Fractionation of the lipids

The lipids were fractionated on columns of silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Co., Williamsport, PA) which were 1 cm in diameter and contained 1 g dry weight (wt.). The columns were packed with a chloroform slurry and the lipids applied to the columns in chloroform. The neutral lipids were eluted with 10 mL of chloroform; the glycolipids which contain the PHB were eluted with 10 mL of acetone; and the polar lipids were eluted with 10 mL of methanol. The recovery was shown to be quantitative (King et al. 1977).



SCHEME 1. Analytical sequence used in the analysis of the surface and subsurface aquifer sediment microbiota. TLC, thin-layer chromatography; cGLC, capillary gas–liquid chromatography; HF, cold concentrated hydrofluoric acid hydrolysis; MS, mass spectrometry; PHB, poly-β-hydroxybutyrate; PLFA, phospholipid ester-linked fatty acids; FAME, fatty acids methyl esters; OH-FAME, hydroxy FAME. References to methods are as follows: A, Wilson, McNabb, Balkwill et al. 1983; B, White, Davis et al. 1979; C, Findlay et al. 1985; D, Gehron and White 1982; E, Findlay and White 1983; F, Guckert et al. 1985; G, Gehron and White 1983; H, Gehron et al. 1984; I, Fazio et al. 1982. Dotted lines show ^{14}C-labelled samples.

Analysis of the lipids

The neutral lipid fraction was analyzed for glycerol by capillary gas–liquid chromatography (cGLC) as a measure of glyceride (Gehron and White 1982). The PHB can be recovered quantitatively from the glycolipid fraction (unpublished data). PHB was derivatized by ethanolysis and analyzed by gas–liquid chromatography (GLC) (Findlay and White 1983). The polar lipids were first subjected to mild alkaline methanolysis which yielded the fatty acid methyl esters (FAME) that were in ester linkage with the polar lipids (White, Davis et al. 1979). The FAME were purified by thin-layer chromatography and analyzed by cGLC (Bobbie and White 1980; Guckert et al. 1985). Structures were assigned to the FAME on the basis of retention time compared with standards, the results of hydrogenations, and the fragmentation patterns after gas chromatography – mass spectrometry (MS) (Bobbie and White 1980; Guckert et al. 1985).

Nomenclature of the FAME

Fatty acids are designated by the number of carbon atoms in the aliphatic chain, followed by a colon and the number of double bonds; the position of the double bond nearest the aliphatic (w) end of the molecule is next. The configuration abbreviated as *c* for cis and *t* for trans follows. Suffixes *a*, *i*, *br*, and *cy* indicate anteisobranching, isobranching, branching, and cyclopropane rings, respectively, which are present in the molecule.

Microbial activity

Subsamples of subsurface sandy clay were made with plastic syringes with the ends removed. The plugs of sediment were suspended into a thick slurry with an equal volume of well water containing 2 μCi [1-^{14}C]sodium acetate (56 μCi/μmol) (1 Ci = 37 GBq)) in a screw-cap test tube; the suspension was mixed and allowed to incubate for 20 min at 25°C. Chloroform–methanol (1:2, v/v) was then added to a final proportion of chloroform–methanol-water of 1:2:0.8 (by volume). After an extraction of at least 2 h, the chloroform–methanol was decanted into a separatory funnel and the residue washed 3 times with a total volume of chloroform equal to half the total methanol. Sufficient water containing 2 m*M* NaOH was added to form two phases with a final composition of chloroform–methanol–water of 1:1:0.9 (by volume). After separation had proceeded for at least 12 h, the chloroform was recovered and fractionated on disposable silicic acid columns made with pasteur pipettes and the radioactivity in the PHB and PLFA determined. This is a modification of the method published by Findlay et al. (1985). The nuclides and scintillation fluors were supplied by New England Nuclear Corp., Boston, MA.

Analysis of the lipid-extracted residue

The lipid-extracted residue was divided and a portion methylated in the presence of anhydrous methanol – concentrated HCl – chloroform (10:1:1, by volume). The methyl esters were reduced in the presence of sodium borodeuteride, the polymers dialyzed, hydrolyzed, derivatized, and analyzed for carbohydrates and uronic acids as described (Fazio et al. 1982). The second portion of the lipid-extracted residue was hydrolyzed with concentrated hydrofluoric acid at 0°C, followed with a second acid hydrolysis, and after purification and derivatization was analyzed for teichoic acid glycerol (Gehron et al. 1984). In some cases a third portion of the lipid-extracted residue was hydrolyzed and extracted for covalently bound fatty acids in the lipopolysaccharide – lipid A of the gram-negative bacteria (Parker et al. 1982).

A diagram of the analytical flow sheet for the surface and subsurface aquifer sediments is illustrated in Scheme 1.

Statistics

The data were analyzed using an analysis of variance (ANOVA) program BMDPO8V and the SPSS DISCRIMINANT on the Florida State University Cyber 760 computer.

Results

Biomass of the surface and subsurface microbiota

Results of the analysis of the surface and subsurface sandy clay sediments from the site at Lula, OK, for various measures

TABLE 1. Biomass estimates of surface and subsurface soil microbiota (Lula, OK, site)

Component	Surface (0–10 cm)*	Subsurface (4 m)
Dry weight, nmol/g		
Phospholipid	4.18(0.31)	0.18(0.03)*b*
Glycolipid (glycerol)	1.16(0.48)	0.38(0.14)*a*
Neutral lipid (glycerol)	3.84(0.94)	0.38(0.14)*b*
Teichoic acid (glycerol)	37.3(13.8)	6.3(2.7)*b*
PHB	2.54(0.76)	2.74(0.58)
Total lipid hydroxy fatty acids	0.11(0.01)	0.2(0.01)
Total polymer carbohydrate	223(84)	7.6(4.6)*a*
Ratio of component to phospholipid		
Glycolipid (glycerol)	0.4(0.1)	3.6(1.8)*a*
Neutral lipid (glycerol)	0.9(0.2)	7.1(3.5)*b*
Teichoic acid (glycerol)	8.8(3.5)	56(13)*a*
PHB	0.6(0.09)	9.1(1.00)
Total lipid hydroxy fatty acids	0.03(0.01)	1.8(0.1)*a*
Total polymer carbohydrate	53(5.8)	69(30)

NOTE: Data are given as mean (standard deviation), $n = 3$. Significance betweeen means (logarithms of ratios) by one-way ANOVA are indicated by *a* ($P < 0.05$) and *b* ($P < 0.001$). PHB, poly-β-hydroxybutyrate.
*Depth.

of microbial biomass and extracellular polysaccharide polymers are given in Table 1. The absolute values of the extractable phospholipid, the glycolipid glycerol, the neutral lipid glycerol, the teichoic acid glycerol, and the total polymer carbohydrate are significantly greater in the surface microbiota. The PHB and total lipid hydroxy fatty acids are not significantly different between the surface and subsurface sediments. Comparison of these components relative to the total cellular biomass estimated as the extractable phospholipid shows the glycolipid glycerol, neutral lipid glycerol, teichoic acid glycerol, PHB, and lipid hydroxy fatty acids are significantly higher in the microbiota of the subsurface sediments.

Exopolymer polysaccharide concentration

A portion of the residue after lipid extraction was analyzed for polymer polysaccharide (Table 2). Seven sugars were identified. The presence of high proportions of glucuronic and galacturonic acids was confirmed by cGC–MS but poor recovery precluded quantitative estimates. There was clearly a much higher polymer carbohydrate content in the surface soils than in the subsurface sediments. The proportions of the monomers in the surface and subsurface both showed highest content of glucose, with mannose as the sugar present in next highest proportions. Arabinose is present in the third highest proportions in the subsurface sediments in contrast to galactose in the surface microbiota.

Community structure of the microbiota

The ester-linked fatty acids of the phospholipids can be utilized to provide insight into the community stucture of the microbiota (White 1983). Analysis of the proportions of the major branched, saturated, and unsaturated fatty acids ester-linked to the phospholipids from the surface and subsurface sandy clays from the site at Lula, OK, a subsurface sandy clay from Conroe, TX, and a subsurface limestone from England, are given in Table 3. The subsurface unconsolidated materials contain lower concentrations of fatty acids than the surface microbiota. The subsurface microbiota contain essentially no polyenoic fatty acids with chain lengths longer than 19 carbon

TABLE 2. Surface and subsurface exopolymer polysaccharide composition (in nanomoles per gram dry wt.) (Lula, OK)

Component	Surface (0–10 cm)*	Subsurface (4 m)
Arabinose	14(8)	1.6(1.2)
Xylose	14(12)	0.9(0.9)
Fucose	27(13)	0.7(0.3)
Rhamnose acid	37(15)	0.6(0.5)
Glucose	73.4(62)	2.8(2.4)
Galactose	45(13)	1.1(1.6)
Mannose	59(53)	1.8(0.4)

NOTE: Data are given as mean (standard deviation), $n = 4$.
*Depth.

atoms. Differences in composition are more readily analyzed if the proportions of the fatty acids are examined. Major differences in proportions of fatty acids can be seen in the data of Table 3. The high variance reflects the patchy distribution of microbiota.

The success of stepwise discriminant analysis in showing the effects of epibenthic predators at the top of a mud flat food chain on microbiota at the base of the food chain (Federle et al. 1983) suggested use of this technique with the subsurface groundwater microbiota. Table 4 lists the fatty acids that contribute most to the discrimination between samples taken from the surface and subsurface sediments at the uncontaminated site at Lula, OK, the subsurface sediments contaminated with creosote waste from Conroe, TX, and the subsurface limestone from England. The order of entry of the fatty acids into the analysis indicates those variables that have the greatest discrimination power. The "*F* to remove" indicates the penalty for removal of each fatty acid from the analysis. The discriminant coefficient *Z* relates to the weighting of that variable in the discrimination. The greater the absolute value of these functions, the greater the contribution to the discrimination. Figure 1 is a graphical representation of these data. The high proportions of *a* 15:0 and *i* 16:0, together with the low proportions of 16:1w7*c*, contribute most to the clear distinction between the three subsurface sites and the surface soil (top of Fig. 1, Table 4). The high proportions of *a* 15:0 and *i* 16:0 in the subsurface limestone clearly distinguish it from the two subsurface sandy clay sediments. Different proportions of 18:1w9*c* and 18:0 differentiate between the two subsurface sandy clay sites.

Effects of creosote waste contamination on the subsurface aquifer microbiota

Contamination of the subsurface clay in the vadose zone induces changes in the total microbial biomass as measured by the extractable lipid phosphate (Table 5). The structure of this community appeared to shift from a gram-positive microbiota rich in glycerol teichoic acid to one with a much smaller proportion of the total biomass containing teichoic acid. There was a large variance in these measurements. In data not shown, the proportions of lipopolysaccharide – lipid A hydroxy fatty acids from gram-negative bacteria is higher where contamination exists. This analysis is complicated by the low recoveries of hydroxy fatty acids from sediments containing clay.

The metabolic activity, measured by the incorporation of [^{14}C]acetate into the PLFA after 20 min incubation, appeared to increase in the contaminated samples (data not shown). During conditions of unbalanced growth, microorganisms can accumu-

TABLE 3. Proportions of phospholipid ester-linked fatty acids of surface, subsurface, control, and two contaminated samples given as percent of total phospholipid ester-linked FAME

		Conroe, TX		Lula, OK		
Fatty acid	Control (6 m)*	Contaminated (6 m)	Most contaminated (6 m)	Surface (0–10 cm)	Subsurface (4 m)	English limestone (35 m)
14:0	<0.1*a*	<0.1	<0.1	1.0(1.0)	1.1(2.0)	3.6(2.1)
i 15:0	<0.1*a*	<0.1	<0.1	9.2(2.1)	<0.1	1.2(1.0)
a 15:0	<0.1*a*	<0.1	<0.1	<0.1	<0.1	1.4(1.1)
15:0	<0.1*a*	<0.1	<0.1	6.2(1.0)	1.2(1.0)	1.9(1.3)
i 16:0	<0.1*a*	<0.1	<0.1	5.3(2.8)	<0.1	1.3(0.9)
16:1w7*c*	<0.1*a*	<0.1	<0.1	3.2(1.1)	2.2(3.0)	9.1(2.4)
16:0	36	62(12)	55(12)	16(4)	37(8)	31(3.5)
br 16:0	<0.1	<0.1	<0.1	<0.1	<0.1	6.5(5.8)
i 17:0	<0.1*a*	<0.1	<0.1	<0.1	<0.1	0.9(0.5)
a 17:0	<0.1*a*	<0.1	<0.1	<0.1	<0.1	2.4(1.2)
17:0	<0.1	<0.1	4.0(6.0)	6.2(7.7)	3.1(1.0)	1.8(0.4)
18:2w6	38	14(8.0)	15(16)	3.1(3.3)	2.3(1.4)	<0.1
18:1w9*c*	13*a*	10(4)	13(0.8)	8.1(11)	9.4(2.2)	9.8(0.4)
18:1w7*c*	2.1	1.0(1.4)	2.4(2.5)	13(4.1)	1.2(1.0)	6.6(0.4)
18:0	4.0*a*	5.2(1.1)	5.1(2.1)	8.1(1.2)	37(11)	11(1.9)
cy 19:0	<0.1	<0.1	<0.1	<0.1	<0.1	1.9(1.2)
20:0	<0.1	<0.1	<0.1	1.0(0.3)	2.1(2.0)	1.2(0.2)
22:1w9*c*	<0.1	<0.1	<0.1	<0.1	<0.1	1.2(1.2)
22:0	1.1*a*	1.0(1.2)	1.0(1.1)	<0.1	2.6(0.8)	3.9(1.0)
% of total	94	93	95	80	97	95

NOTE: Data are given as mean (standard deviation), $n = 4$. *a* indicates significant differences between means ($P < 0.05$) by ANOVA.
*Depth.

TABLE 4. Major difference in proportions of ester-linked fatty acids from the phospholipids of surface soil and subsurface sediment which contribute to the distinction between a surface soil, a subsurface limestone, and two subsurface clays as measured by stepwise discriminant analysis of the 14 components which show significant differences ($P < 0.05$) by ANOVA from the data summarized in Table 3

Fatty acid[a]	*F* to remove[b]	Discriminant function (*Z*)[c]
Surface soil, subsurface limestone, and subsurface clays (two sites)		
14:0	14.3	5
i 15:0	169	−27.4
16:1w7t	698	30.4
22:0	1.3	2.0
% of total variance		99.9
Subsurface limestone and subsurface clays (two sites)		
a 15:0	71	14.8
i 16:0	206	15.2
16:1w7c	1	0.8
22:0	1.7	0.9
% of total variance		99.9
Subsurface clays (two sites)		
18:1w9c	13	−2
18:0	37	2.1
% of total variance		99.9

[a]Variables listed in the order of entry into the discriminant analysis. Variables not listed did not contribute to the discrimination.
[b]*F* to remove indicates the penalty for the removal of the variable from the discriminant function.
[c]The standardized coefficient (*Z*) with the largest absolute values contribute most to discrimination by an individual discriminant function.

late ^{14}C in PHB (Nickel et al. 1979). The ratio of the rates of ^{14}C incorporation between PLFA and PHB has been shown to be a sensitive measure of the nutritional status of bacteria in sediments and in the rhizosphere (Findlay et al. 1985; Tunlid et al. 1985). Contamination with creosote waste appeared to shift the subsurface microbiota into more active growth than in uncontaminated sediment as reflected in the higher ratios of PLFA/ PHB (16, 29, and 22 for control, contaminated, and most contaminated, respectively).

Discussion

Sparseness of the subsurface microbiota

The samples recovered 4 m below the surface from uncontaminated sandy clay sediments contain a distinctly different microbiota from that found in surface soils (Tables 1 and 2). The subsurface microbiota are sparse. Based on the extractable phospholipid phosphate and a conversion of 50 μmol lipid phosphate per gram dry wt. (White, Davis et al. 1979) and 10^{12} organisms per gram dry wt., the subsurface microbiota from this site contains 2.2×10^6 microbes the size of *Pseudomonas fluorescens* per gram dry wt. This compares with 8×10^7 per gram dry wt. in the dry clays at the surface of the Lula site or 10^9 usually found in richer surface soils and sediments (Gehron and White 1983). These calculations agree with estimates based on the extractable phospholipids and on the muramic acid measurements from the cell walls (Gehron and White 1983; Gehron et al. 1984).

Community structure of the subsurface microbiota

There is a strikingly higher proportion of gram-positive bacteria based on the teichoic acid glycerol per phospholipid in the subsurface microbiota when compared with surface soils (Table 1). This confirms earlier work that showed higher proportions of gram-positive bacteria based on teichoic acid in

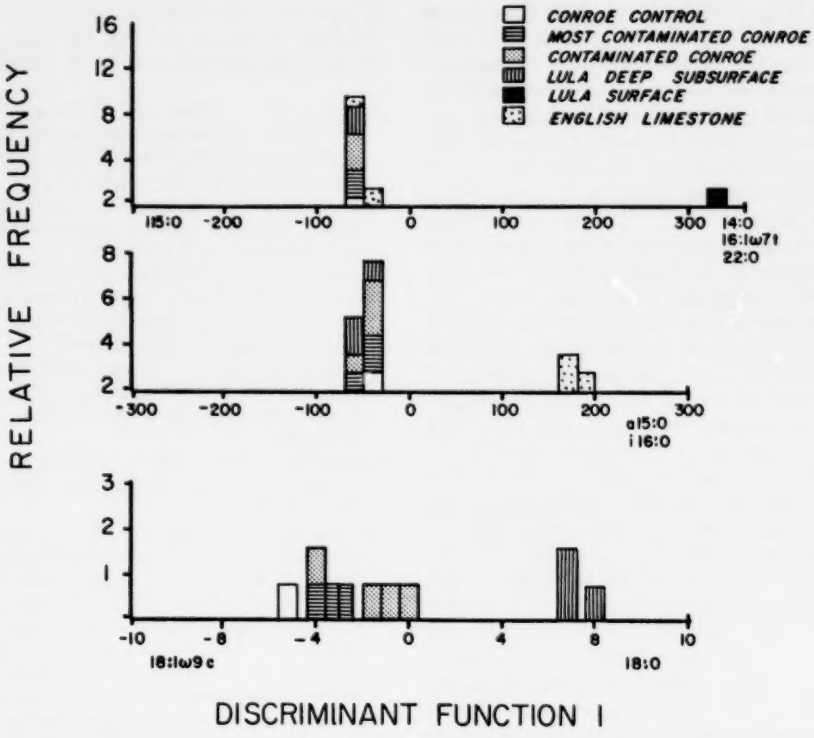


FIG. 1. Histograms of the stepwise discriminant analysis of ester-linked phospholipid fatty acids from the surface, subsurface sandy clays (two sites), and subsurface limestone (upper panel); from the subsurface sandy clays (two sites) and the limestone (middle panel); and from the two subsurface sandy clays (lower panel). Fatty acids with the greatest contribution to the discrimination between sediments are indicated on the axis (see Table 4 for values).

TABLE 5. Effects of contamination with phenols on the subsurface clay microbiota (Conroe, TX)

	Control	Contaminated	Most contaminated
Biomass			
Total extractable phospholipid	0.6(0.5)	1.0(0.8)	1.9(0.5)
Community structure			
Teichoic acid glycerol	33(40)	21(30)	10(2.4)
Ratio, teichoic acid/phospholipid	56(59)	20(34)	5.4(1)

NOTE: No significant difference ($P < 0.05$) between means was demonstrated by ANOVA ($n = 4$). Data are given in nanomoles per gram dry weight.

soils than in sediments and in deeper compared with shallow soils (Gehron et al. 1984). The higher proportions of gram-positive bacteria proved particularly important as contamination of the subsurface in one well-studied case shows a progressive diminution in the proportion of gram-positive bacteria with increasing activity in the aerobic biodegradation of creosote waste (Table 5, Smith et al. 1985).

The finding of a high proportion of gram-positive bacteria also emphasizes the value of quantitative biochemical assays of subsurface environmental sediments. Studies of culturable subsurface microbiota have yet to show the predominance of gram-positive organisms (D. L. Balkwill, personal communication). Studies of the organisms isolated with great care from the groundwater itself also fail to show the high proportions of gram-positive bacteria that characterize the uncontaminated subsurface environment (Hirsch and Rades-Rohkohl 1983).

The high proportions of gram-positive bacteria in the uncontaminated subsurface microbiota are confirmed by the high levels of glycolipid glycerol (Table 1). Gram-positive bacteria contain high levels of glycosyl diglyceride lipids (Shaw 1970, 1974).

Microeukaryotes

Recent studies (J. L. Sinclair and W. C. Ghiorse. 1985. Abstr. Annu. Meet. Am. Soc. Microbiol.) indicate that amoeboid protozoa are present in groundwater sediment. The presence of glyceride, measured as the neutral lipid glycerol

(Table 1) (Gehron and White 1982), most probably represented the presence of diglycerides from the breakdown of bacterial phospholipids rather than the triglycerides from microeukaryotes. Recent experiments have shown the rapid loss of PLFA with an increase in neutral lipid fatty acids in subsurface soils incubated at ambient temperature for several hours (unpublished data). Phospholipids, which are a much better measure of the active cellular biomass (White, Davis et al. 1979), show microeukaryotes must be sparse and contain an unusual fatty acid pattern in their phospholipids. One of the characteristic features of every aseptically recovered sediment we have examined has been the relative lack of polyenoic fatty acids longer than 19 carbon atoms from the phospholipid fraction when compared with estuarine or abyssal marine sediments (White et al. 1984; White et al. 1985; Baird et al. 1985).

The subsurface microbiota contain fewer total microorganisms, with no microeukaryotes contributing polyenoic fatty acids longer than 19 carbon atoms, as well as, when uncontaminated, a higher relative proportion of gram-positive bacteria than surface soils or sediments. Possibly a more sensitive test would be to show a distinctive subsurface microbiota by analyzing the proportion of branched, saturated, and unsaturated phospholipid ester-linked fatty acids (Tables 3 and 4, Fig. 1). Using the stepwise discriminant analysis, it is possible to define the specific fatty acids that most contribute to the differentiation between surface and subsurface soils (Table 4, Fig. 1, top panel), between subsurface sandy clays and limestone (upper middle panels), and between subsurface sandy clays between two sites (lower middle panels). As information about the specific fatty acid profiles of subsurface organisms in monoculture becomes available, interpretation of the shifts in microbial ecology which correspond to fatty acid differences becomes possible. This approach has been taken by researchers studying the effects of light or surface structure on the marine microfouling community (Nickels et al. 1981; Berk et al. 1981).

Effects of contamination on the subsurface microbiota

Studies by J. T. Wilson and colleagues have shown that contamination of subsurface sandy clays increases the biodegradatory activity of the microbiota (Wilson and McNabb 1983; Wilson, McNabb, Wilson et al. 1983). The increase in biodegradatory activity at the creosote contaminated Conroe, TX, site (Wilson, McNabb et al. 1985; Wilson, Miller et al. 1985) is paralleled by an increase in total microbial biomass as reflected in the extractable phospholipid (Table 5). There is a progressive decrease in the content of glycerol teichoic acid with increasing contamination (Table 5) and in the possible synthesis of PLFA. With additional studies of contaminated sites, it may become possible to derive specific indices that may be useful in predicting the biodegradative potential of subsurface aquifer sediments.

Disclaimer

Although the research described in this article has been funded wholly or in part by the United States Environmental Protection Agency, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

Acknowledgements

This work was supported by collaborative agreement CR-80-9994 from the Environmental Protection Agency administered by the Robert S. Kerr Environmental Research Laboratory, Ada, OK. Thanks are due to R. H. Findlay for some of the radioactive measurements and to M. B. Trexler for the illustrations.

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